EXHIBIT 1

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…guish•a•ble (ĭn'ĭk-sting'gwĭ-shə-bəl) *adj.* Difficult or …guish: *an inextinguishable flame; an inextinguishable …*

…guish•a•bly *adv.*

…•ble (ĭn'ĭk-stûr'pə-bəl) *adj.* Difficult or impossible to …rrect.

…mis (ĭn ĕk-strē'mĭs) *adv.* **1.** At the point of death. **2.** In …cumstances. [Latin *in extrēmīs* : *in*, in + *extrēmīs*, ab… extreme.]

…•ate (ĭn-ĕk'strĭ-kə-bəl, ĭn'ĭk-strĭk'ə-bəl) *adj.* **1a.** So …ed as to make escape impossible: *an inextricable maze; …s of deceit.* **b.** Difficult or impossible to disentangle or …tangle of threads. **c.** Too involved or complicated to …problem. **2.** Unavoidable; inescapable: *bound togeth… fate.* **—in•ex'tri•ca•bil'i•ty, in•ex'tri•ca•ble• …'tri•ca•bly** *adv.*

…-range nuclear forces

…ry **2.** inferior **3.** infinitive **4.** infinity

…•ble (ĭn-făl'ə-bəl) *adj.* **1.** Incapable of erring: *an infallible …urce of information.* **2.** Incapable of failing; certain: *… an infallible rule.* **3.** *Roman Catholic Church* Inca… …ounding doctrine on faith or morals. [Middle Eng… Latin *infallibilis* : Latin *in-*, not; see IN–[1] + Medieval …ible; see FALLIBLE.] **—in•fal'li•bil'i•ty, in•fal'li• …fal'li•bly** *adv.*

…•mous (ĭn'fə-məs) *adj.* **1.** Having an exceedingly bad reputa… **2.** Causing or deserving infamy; heinous: *an infamous …* Punishable by severe measures, such as death, long im… of civil rights. **b.** Convicted of a crime, such as trea… …ries such a punishment. [Middle English *infamis*, … *in-*, not; see IN–[1] + *fāma*, renown, fame; see **bhā-**[2] …'fa•mous•ly** *adv.* **—in'fa•mous•ness** *n.*

…ous) *n., pl.* **-mies** **1.** Evil fame or reputation. **2.** The …infamous. **3.** An evil or criminal act that is publicly …nown: *infamie*, dishonor, from Old French, from Latin …mis, infamous. See INFAMOUS.]

…sē) *n., pl.* **-cies** **1.** The earliest period of childhood, …the ability to walk has been acquired. **2.** The state of … **3.** An early stage of existence: *Space exploration is still …* The state or period of being a minor.

… **1.** A child in the earliest period of life, especially …ore walk. **2.** *Law* A person under the legal age of ma…

… **1.** Of or being in infancy. **2.** Intended for infants … **3.** Newly begun or formed: *an infant enterprise.* [Mid… Old French *enfant*, from Latin *īnfāns, īnfant-*, from … speak, young : *in-*, not; see IN–[1] + *fāns*, present parti… see **bhā-**[2] in Appendix I.]

…fän', -fän'-) *n.* A daughter of a Spanish or Portu… and Portuguese, feminine of *infante*, infante; see IN-…

…'tē, -fän'tä) *n.* A son of a Spanish or Portuguese … heir to the throne. [Spanish and Portuguese, both … *infans-*, infant. See INFANT.]

…fän'tĭ-sīd') *n.* **1.** The act of killing an infant. **2.** … newborn infants. **3.** One who kills an infant. [Late … the killing of a child and *infanticīda*, killer of a child : … infant; see INFANT + Latin *-cidium* and *-cīda*, -cide.]

…'ĭ-sīd'ĭl) *adj.*

…-tĭl', -tīl) *adj.* **1.** Of or relating to infants or in… or suggesting a lack of maturity; childish: *infantile … remark.* [Middle English *infantil*, from Latin *īn… infant-*, infant. See INFANT.]

…ism *n.* Autism.

…alysis *n.* See **poliomyelitis.**

…fən-tĭl-ĭz'əm, ĭn-făn'tl-) *n.* **1.** A state of arrested … adult, characterized by retention of infantile mental… … stunted growth and sexual immaturity, and often by … immaturity, as in behavior or character: *"infan… … of so much American cinema and culture"* (John … act or remark.

…n-tl-īz', ĭn-făn'-) *tr.v.* **-ized, -iz•ing, -iz•es** **1.** … state or condition: *"It creates a crisis that infan… … men to squabble like kids about trivial things"* … or condescend to as if still a young child: *"The … …tized his patient"* (Judith Moore). **—in•fan'…**

…-tīn', -tīn) *adj.* Infantile; childish.

…sē) *n., pl.* **-tries** **1.** The branch of an army made … fight on foot. **2.** Soldiers armed and trained to … *ordered his infantry to attack.* **3.** A unit, such … soldiers: *Company B of the 7th Infantry.* [French … French, from Old Italian *infanteria*, from *infante*, … Latin *infāns, infant-*, infant. See INFANT.]

…ing vehicle *n.* A heavily armed, armored combat … wheels and often having amphibious capability, … into battle and support them there.

…fən-trē-mən) *n.* A soldier in the infantry.

… *British* A kindergarten.

…-färkt') *n.* An area of tissue that undergoes ne… …on of local blood supply, as by a thrombus … Latin *infarctus*, past participle of *infarcīre*, to cram : … to stuff.] **—in•farct'ed** *adj.*

in•farc•tion (ĭn-färk'shən) *n.* **1.** The formation or development of an infarct. **2.** An infarct.

in•fat•u•ate (ĭn-făch'ōō-āt') *tr.v.* **-at•ed, -at•ing, -ates** **1.** To inspire with unreasoning love or attachment. **2.** To cause to behave foolishly. ❖ *adj.* (-ĭt, -āt') Infatuated. [Latin *infatuāre, infatuāt-* : *in-*, causative pref.; see IN–[2] + *fatuus*, foolish.]

in•fat•u•at•ed (ĭn-făch'ōō-ā'tĭd) *adj.* Possessed by an unreasoning passion or attraction. **—in•fat'u•at'ed•ly** *adv.*

in•fat•u•a•tion (ĭ-făch'ōō-ā'shən) *n.* **1.** A foolish, unreasoning, or extravagant passion or attraction. See synonyms at **love.** **2.** An object of extravagant, short-lived passion.

in•fau•na (ĭn'fô'nə) *n.* Aquatic animals that live in the substrate of a body of water, especially in a soft sea bottom. [IN–[2] + FAUNA.]

in•fea•si•ble (ĭn-fē'zə-bəl) *adj.* Not feasible; impracticable. **—in• fea'si•bil'i•ty** *n.* **—in•fea'si•bly** *adv.*

in•fect (ĭn-fĕkt') *tr.v.* **-fect•ed, -fect•ing, -fects** **1.** To contaminate with a pathogenic microorganism or agent. **2.** To communicate a pathogen or disease to. **3.** To invade and produce infection in. **4.** To contaminate or corrupt: *envy that infected their thoughts; a society that was infected by racism.* **5.** To affect in a contagious way: *"His fear infected me, and . . . I followed as fast as I could"* (W.H. Hudson). [Middle English *infecten*, to afflict with disease, from Latin *īnficere, infect-*, to stain, infect (*in-*, in; see IN–[2] + *facere*, to do; see **dhē-** in Appendix I).]

in•fec•tion (ĭn-fĕk'shən) *n.* **1a.** Invasion by and multiplication of pathogenic microorganisms in a bodily part or tissue, which may produce subsequent tissue injury and progress to overt disease through a variety of cellular or toxic mechanisms. **b.** An instance of being infected. **c.** An agent or a contaminated substance responsible for one's becoming infected. **d.** The pathological state resulting from having been infected. **2.** An infectious disease. **3a.** Moral contamination or corruption. **b.** Ready communication of an emotion or attitude by contact or example.

in•fec•tious (ĭn-fĕk'shəs) *adj.* **1.** Capable of causing infection. **2.** Caused by or capable of being transmitted by infection. **3.** Caused by a pathogenic microorganism or agent. **4.** Easily or readily communicated: *an infectious laugh.* **—in•fec'tious•ly** *adv.* **—in•fec'tious•ness** *n.*

infectious enterohepatitis *n.* See **blackhead** (sense 2).

infectious hepatitis *n.* See **hepatitis A.**

infectious mononucleosis *n.* A common, acute, infectious disease, usually affecting young people, caused by Epstein-Barr virus and characterized by fever, swollen lymph nodes, sore throat, and lymphocyte abnormalities. Also called *glandular fever.*

in•fec•tive (ĭn-fĕk'tĭv) *adj.* Capable of producing infection; infectious. **—in•fec'tive•ness, in'fec•tiv'i•ty** *n.*

In•fe•lic•i•tous (ĭn'fĭ-lĭs'ĭ-təs) *adj.* **1.** Inappropriate; ill-chosen: *an infelicitous remark.* **2.** Not happy; unfortunate. **—in'fe•lic'i•tous•ly** *adv.*

in•fe•lic•i•ty (ĭn'fĭ-lĭs'ĭ-tē) *n., pl.* **-ties** **1.** The quality or condition of being infelicitous. **2.** Something inappropriate or unpleasing. [Middle English *infelicite*, from Latin *infēlīcitās*, from *infēlīx, infēlīc-*, unhappy : *in-*, not; see IN–[1] + *fēlīx*, happy; see **dhē(i)-** in Appendix I.]

in•fer (ĭn-fûr') *v.* **-ferred, -fer•ring, -fers** —*tr.* **1.** To conclude from evidence or premises. **2.** To reason from circumstance; surmise: *We can infer that his motive in publishing the diary was less than honorable.* **3.** To lead to as a consequence or conclusion: *"Socrates argued that a statue inferred the existence of a sculptor"* (Academy). **4.** To hint; imply. —*intr.* To draw inferences. [Latin *inferre*, to bring in, adduce : *in-*, in; see IN–[2] + *ferre*, to bear; see **bher-**[1] in Appendix I.] **—in•fer'a•ble** *adj.* **—in•fer'a•bly** *adv.* **—in•fer'rer** *n.*

Usage Note *Infer* is sometimes confused with *imply*, but the distinction is a useful one. When we say that a speaker or sentence implies something, we mean that it is conveyed or suggested without being stated outright: *When the mayor said that she would not rule out a business tax increase, she implied* (not *inferred*) *that some taxes might be raised.* Inference, on the other hand, is the activity performed by a reader or interpreter in drawing conclusions that are not explicit in what is said: *When the mayor said that she would not rule out a tax increase, we inferred that she had been consulting with some new financial advisers, since her old advisers were in favor of tax reductions.*

in•fer•ence (ĭn'fər-əns) *n.* **1a.** The act or process of deriving logical conclusions from premises known or assumed to be true. **b.** The act of reasoning from factual knowledge or evidence. **2a.** Something inferred. **b.** *Usage Problem* A hint or suggestion: *The editorial contained an inference of foul play in the awarding of the contract.* See Usage Note at **infer.**

in•fer•en•tial (ĭn'fə-rĕn'shəl) *adj.* **1.** Of, relating to, or involving inference. **2.** Derived or capable of being derived by inference. **—in'fer•en'tial•ly** *adv.*

in•fe•ri•or (ĭn-fîr'ē-ər) *adj.* **1.** Low or lower in order, degree, or rank: *Captain is an inferior rank to major.* **2a.** Low or lower in quality, value, or estimation: *inferior craft; felt inferior to his older sibling.* **b.** Second-rate; poor: *an inferior translation.* **3.** Situated under or beneath. **4.** *Botany* Located below the perianth and other floral parts. Used of an ovary. **5.** *Anatomy* Located beneath or directed downward. **6.** *Printing* Set below the normal line of type; subscript. **7.** *Astronomy* **a.** Orbiting between Earth and the sun: *Mercury is an inferior planet.* **b.** Lying below the horizon. ❖ *n.* **1.** A person lower in rank, status, or accomplishment than another. **2.** *Printing* An inferior character, such as the number 2 in CO_2. [Middle English, from Latin *inferior*, comparative of *inferus*, low. See **ndher-** in Appendix I.] **—in•fe'ri•or'i•ty** (-ôr'ĭ-tē, -ŏr'-) *n.* **—in• fe'ri•or•ly** *adv.*

inferiority complex *n.* A persistent sense of inadequacy or a ten-

ă pat	oi boy
ā pay	ou out
âr care	ŏŏ took
ä father	ōō boot
ĕ pet	ŭ cut
ē be	ûr urge
ĭ pit	th thin
ī pie	*th* this
îr pier	hw which
ŏ pot	zh vision
ō toe	ə about, item
ô paw	♦ regionalism

Stress marks: ' (primary); ˌ (secondary), as in **dictionary** (dĭk'shə-nĕr'ē)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Robert MARTUZA *et al.*
Title:	REPLICATION-COMPETENT HERPES SIMPLEX VIRUS MEDIATES DESTRUCTION OF NEOPLASTIC CELLS
Appl. No.:	10/748,233
Filing Date:	12/31/2003
Examiner:	Wu Cheng Winston Shen
Art Unit:	1632
Confirmation Number:	7116

DECLARATION UNDER 37 CFR § 1.132

Commissioner for P[illegible]nts
P.O. Box 1450
Alexandria, VA 22[illegible]3-1450

Sir:

I, Samuel D. Rabkin, hereby declare that:

1. I am a co-inventor of U.S. patent application serial No. 10/748,233 ("the application"), which, I understand, was filed on December 31, 2003, and benefits from a priority date of June 23, 1994.

2. I received my Bachelor of Science in Biology from University of Toronto (Toronto, Canada) in 1976. In 1978, I received my Master of Science in Microbiology from Hebrew University of Jerusalem (Jerusalem, Israel). Since then I have worked in the area of herpes simplex virus and its application for treating cancer. In 1983 I received my Ph.D. in Microbiology from University of Chicago (Chicago, IL). I am currently an associate professor of the Harvard Medical School and an associate virologist of Massachusetts General Hospital.

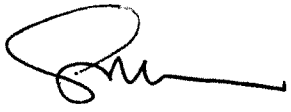
3. I understand that subject matter claimed in the application ("the claimed invention") is deemed by the U.S. Patent and Trademark Office to have been presaged by the contemporaneous literature and, hence, is unpatentable.

4. I have reference to evidence from the pre-filing literature, however, demonstrating that cytokines such as IL-1α, IL-2, IL-3, TNF, IFN-α, IFN-β, IFNγ, M-CSF-1 and GM-CSF were expected to have antiviral effects, such as protecting a host from herpes simplex virus (HSV) infection and preventing HSV replication in the host. In particular, Chatterjee *et al., J. Virol.* 56: 419-425 (1985), Exhibit A, show that IFN-α2 and IFN-β block HSV-1 replication. Ito *et al., Lymphokine Res.* 6: 309-318 (1987), abstract submitted herewith as Exhibit B, describe that tumor necrosis factor (TNF), alone or in combination with IFNγ, has antiviral activities against human herpes simplex virus (HSV) types 1 and 2, e.g., suppression of virus growth. Feduchi *et al., J. Virology* 63: 1354-59 (1989), Exhibit C, confirm that TNF and IFNγ exhibit synergistic inhibitory effects on HSV-1 replication. Chatterjee *et al., Virus Research* 12: 33[illegible]2 (198[illegible] Exhibit D, report that human alpha interferon (IFN-α) A/D significantly reduces the replication and cell fusion induced by HSV-1. Kohl *et al., J. Infect. Dis.* 159: 23[illegible]47 (1989), abstract submitted as Exhibit E, show that interleukin-2 (IL-2) protects neonatal mice from HSV infection. Gasbarrini *et al., J. Interferon Res.* 9: 227-37 (1989), abstract submitted as Exhibit F, disclose that IFN-α B/D is highly effective in preventing viral replication and cell destruction induced by HSV-1 in human monocyte cultures. Iida *et al., Vaccine* 7: 229-33 (1989), Exhibit G, demonstrate that GM-CSF has protective activity against herpes simplex viral infection in mice. Chan *et al., Immunology* 71: 358-63 (1990), Exhibit H, present that IL-3 markedly inhibits HSV-1 replication in primary mouse embryonic head cell cultures. Berkowitz *et al., Arch. Virol.* 124: 83-93 (1992), Exhibit I, conclude that IL-1α, IL-2, M-CSF-1, as well as combinations of IL-2 and M-CSF-1, are effective in protecting mice against HSV-1 infection.

5. In light of the above-mentioned evidence, it is my opinion, as an expert in the field of HSV-mediated therapies, that achieving an enhanced tumor therapy effect by combining an HSV with cytokines would have been unexpected, *circa* June of 1994. Thus, the claimed invention requires a cytokine-expressing HSV to infect and replicate in tumor cells, thereby to elicit an anti-tumor immune response, and yet the contemporaneous literature, which I

have summarized above, indicated that the cytokines would counter or decrease the prerequisite HSV infection and replication.

6. I declare that the statements made herein of my knowledge are true and all statements on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therein.



Samuel D. Rabkin

5/5/08

Date

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Vol. 56, No. 2

Effect of Cloned Human Interferons on Protein Synthesis and Morphogenesis of Herpes Simplex Virus

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Pretreatment of human fibroblast cells with 100 U of either cloned human alpha-2 or beta interferon per ml for 24 h reduced the release of infectious herpes simplex virus type 1 by more than 99%. This inhibition in infectivity correlated well with the total number of extracellular virus particles released from treated cells as determined by DNA dot blot hybridization analysis. Electron microscopic observations of interferon-treated human fibroblast cells clearly demonstrated typical assembly of nucleocapsids inside the nucleus, even though very few mature extracellular particles were seen. Analysis of virus-specific proteins by the immunoblot technique showed that neither species of interferon had a significant inhibitory effect on the synthesis of major nucleocapsid proteins. However, the synthesis of specific glycoproteins (D and B) was drastically reduced or delayed in beta-interferon-treated cells. The results presented in this communication suggest that cloned human interferons block herpes simplex virus morphogenesis at a late stage and inhibit the release of particles from the treated cells.

Herpesvirus infections are among the most common of all human infections, causing a broad spectrum of diseases which range from asymptomatic life threatening with severe disease, herpes simplex encephalitis and neonatal herpes are associated with high mortality and morbidity. Of the five human herpesviruses, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2, respectively) are particularly important because of frequent recurrence with known transmission to susceptible individuals. With the recognition of these viruses as an important cause of human disease, numerous antiviral drugs have been tested as therapeutic agents and shown to be useful in treating some of these infections. However, viral resistance to drugs may occur, although it is of unknown biologic significance at present (6). We recently showed that cloned human alpha and beta interferons (IFN-α and IFN-β, respectively) block HSV-induced cell fusion and plaque formation in human fibroblast cells (5). Fish et al. (7) have also reported that human IFNs are effective against HSV infection, but the specific stage(s) of the virus life cycle which is affected by IFN was not addressed. Little is known about the effect of IFNs on DNA viruses in general and especially the molecular mechanisms by which IFN-treated cells prevent the replication of these viruses. Gloger and Panet (8) have recently reported that naturally produced human IFN-α inhibited the translation of HSV-specific proteins in treated cells. However, at the same time Munoz and Carrasco (15) reported that no major inhibition of HSV protein synthesis occurred in IFN-α-treated cells. In this communication, we demonstrate that both cloned human IFN-α and IFN-β significantly block the release of total extracellular particles from IFN-treated cells. Neither species of IFN had a significant inhibitory effect on the synthesis of major nucleocapsid proteins, but the synthesis of specific glycoproteins was drastically reduced or delayed. Electron microscopic observations of HSV-infected cells pretreated with these IFNs showed typical assembly of nucleocapsids within the nucleus. These results strongly suggest that these IFNs act in an unique manner in HSV-infected cells by blocking HSV-1 replication at a late stage of morphogenesis and preventing the release of virus particles from the treated cells.

MATERIALS AND METHODS

Cell cultures and viruses. Human foreskin (HFS) cells were prepared by published procedures (18). African green monkey kidney (BS-C-1) cells were obtained from the American Type Culture Collection, Rockville, Md. HFS cells were grown in Eagle minimal essential medium containing 10% heat-inactivated fetal calf serum. BS-C-1 cells were grown in medium 199 also supplemented with 10% heat-inactivated fetal calf serum.

The F and MP strains of HSV-1 were kindly provided by B. Roizman, The University of Chicago, Chicago, Ill.

Reagents and radioisotopes. Reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, Calif. $Na^{125}I$ (15.5 mCi/μg of iodine) was purchased from Amersham Corp., Arlington Heights, Ill. [α-^{32}P]dCTP (800 Ci/mmol) and [α-^{32}P]dTTP (800 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Cloned human IFN-α_2 (10^8 U/mg of protein) and IFN-β (10^9 U/mg of protein) were generously provided by J. Schwartz, Schering-Plough Corp., Bloomfield, N.J., and F. McCormick, Cetus Corp., Emeryville, Calif., respectively.

Antisera. Rabbit antiserum to HSV-1 and monospecific rabbit antisera to glycoproteins B and D (gB and gD, respectively) were kindly provided by B. Norrild, University of Copenhagen, Copenhagen, Denmark (23).

Electron microscopy. IFN-treated and untreated HFS cells grown in 60-mm-diameter dishes were processed for electron microscopy after infection with HSV-1. Cells were carefully washed with phosphate-buffered saline and fixed with 1% glutaraldehyde. Samples were postfixed with 1% osmium tetroxide and embedded in an epoxy resin mixture. Thin sections were then stained with uranyl acetate and lead citrate and were examined under a Philips EM 301 electron microscope.

Southern dot blot technique. Viral DNA samples treated

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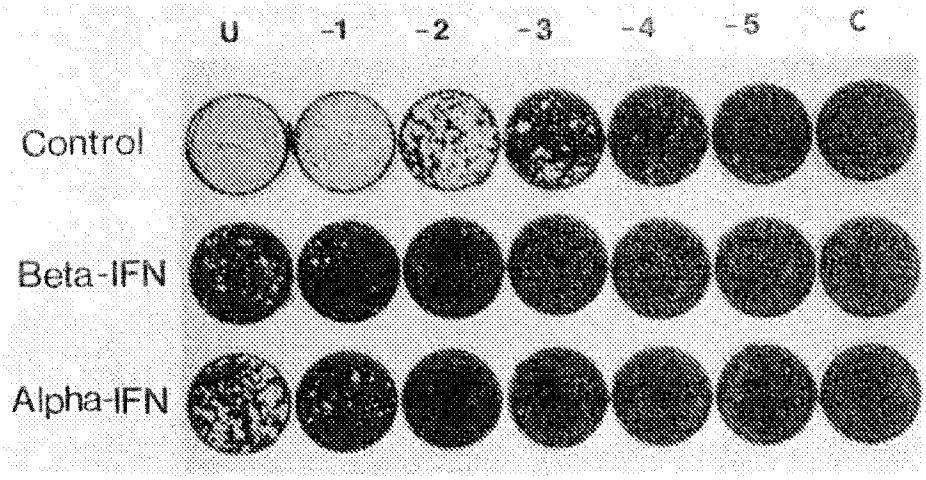


FIG. 1. Effect of cloned human IFNs on replication of HSV-1 in human cells. Human fibroblast cells were pretreated with either IFN-α_2 or IFN-β and then infected with the MP strain of HSV-1 as described in the text. Supernatants were collected, serially diluted, and then tested for their ability to form plaques in BS-C-1 cells. PFU per milliliter were calculated after the cells were stained with May-Grunwald-Giemsa stain as described by Neff and Enders (16).

with RNase A and ribonuclease T_1 were diluted and spotted onto nitrocellulose filter paper after they were denatured as described previously (2, 11, 21). Samples on nitrocellulose paper were then hybridized with ^{32}P-labeled nick-translated HSV-1 DNA (1×10^7 cpm/filter) for 18 h. Nick-translated probe was prepared by the standard procedure (14). The filter paper was washed thoroughly in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C and then exposed to Kodak XR-2 film with a DuPont Cronex Hi-Plus intensifying screen.

Polyacrylamide gel electrophoresis and electrophoretic transfer of proteins to nitrocellulose filter paper (Western blotting). Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9.5%) as described by Chatterjee et al. (3). The fractionated proteins were then electrophoretically transferred (0.2 A for 5 h) to nitrocellulose paper. The procedure of Towbin et al. (22) was followed, with some modifications adapted from the method of Johnson et al. (10). After the transfer, nitrocellulose blots were blocked in Bovine Lacto Transfer Technique Optimizer (5% nonfat dry milk in phosphate-buffered saline with 0.001% sodium azide; BLOTTO) (10) and then reacted with selected antisera for 16 h at 37°C. Bound antibodies were detected by reacting the blots with ^{125}I-protein A for 1 h at 37°C followed by autoradiography as described above. Protein A iodination was carried out by the procedure described by Greenwood et al. (9).

RESULTS

Effect of cloned human IFNs on the replication of HSV-1 in human cells. To quantitatively determine the effect of cloned IFNs on the replication of HSV-1, HFS cells were pretreated with 100 U of either cloned IFN-α_2 or IFN-β per ml for 18 h. One set of cells served as an untreated control. All cells were then infected with the MP strain of HSV-1 (multiplicity of infection, approximately 2). Supernatant harvests were collected 24 h postinfection, and the quantities of infectious virus released from IFN-treated and untreated cells were determined by plaque assay on BS-C-1 cells. The result of such an experiment is shown in Fig. 1. Both IFN-α_2 and IFN-β inhibited the release of infectious HSV-1 particles from HFS cells more than 500-fold.

Cloned human IFNs block the release of total extracellular particles. Since the production of infectious virus was inhibited more than 500-fold by cloned human IFNs, it was necessary to determine whether any extracellular noninfectious particles were formed in the presence of these IFNs. HFS cells were pretreated with 100 U of either IFN-α_2 or IFN-β per ml for 18 h and then infected with the MP strain of HSV-1 (multiplicity of infection, approximately 2). One set of cells was kept as an uninfected control. Supernatant harvests were collected 24 h postinfection and clarified, and the virus was then pelleted by centrifugation at 45,000 rpm for 1 h. Viral DNA was extracted from the IFN-treated and untreated samples, treated with RNase A and ribonuclease T_1, and then processed for the DNA dot blot hybridization assay as described in Materials and Methods. The result of this experiment showed a greater than 90% reduction (calculated from ^{32}P counts of each spot) in the release of total extracellular virus particles (Fig. 2). Identical results were observed when extracellular viral proteins were assayed for by immunoblot (data not shown). Thus the block in replication appears to occur before the release of virus from treated cells.

Effect of cloned IFNs on the assembly of nucleocapsids. Since with several retroviruses IFN blocks the release of assembled virus particles from the plasma membrane (20; S. Chatterjee and E. Hunter, Meet. RNA Tumor Viruses, Cold Spring Harbor Laboratory abstr. no. 110, 1983), we were interested in determining whether a similar phenomenon occurred in HSV-infected cells. HFS cells were treated with 100 U of either cloned IFN-α_2 or IFN-β per ml and then infected with the MP strain of HSV-1 as in the previous experiment. The cells were processed for electron microscopy at 24 h postinfection as described in Materials and Methods. The electron micrographs showed the presence of assembled HSV cores inside the nuclei of both the IFN-treated and untreated cells (Fig. 3). However, while in the untreated cells numerous extracellular particles (Fig. 3a) could be observed, only a very few extracellular viral particles in IFN-α-treated cells and only intranuclear cores in IFN-β-treated cells were seen (Fig. 3b and c). These observations correlate well with the results of the DNA dot blot hybridization experiments described previously (5) (Fig.

2) and support the hypothesis that both of these IFNs block HSV-1 replication at a late stage in morphogenesis and in some way inhibit the exit of viral cores from the nucleus of treated cells.

Synthesis of virus-specific proteins in IFN-treated human cells. To determine whether the block observed in virus replication could be correlated with a reduction in the synthesis of specific viral coded proteins and thus prevent normal virus assembly and transport, immunoblotting experiments were performed as described below. Monolayers of HFS cells were treated with 100 U of either IFN-α_2 or IFN-β per ml for 18 h and then infected with the MP strain of HSV-1 as before. One set of cells served as an uninfected control. Cell lysates collected at 18 and 48 h postinfection were processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent immunoblotting as described in Materials and Methods. The blots from this experiment were reacted with rabbit anti-HSV-1 antiserum and ^{125}I-protein A sequentially, and the resulting autoradiograms are displayed in Fig. 4a. Although delay in the appearance of some viral proteins was observed at 18 h postinfection in IFN-β-treated cells, essentially all the major capsid proteins of HSV were synthesized by 48 h postinfection in this set. Indeed, in other experiments, in which the F strain of HSV-1 was used, less significant differences were seen between untreated and IFN-β-treated samples (Fig. 4b). Thus, while these results show that IFN-β slightly delayed the synthesis of HSV-specific proteins in human fibroblast cells, it is clear that by 48 h a majority of the capsid proteins were being synthesized normally. Since it was possible that the extended incubation time (48 h) after HSV infection had increased the level of replication of HSV in the IFN-treated cells, parallel plaque assays were carried out to quantitate the amount of infectious virus released in this experiment. The supernatants collected at 18 and 48 h postinfection from the plates (before lysis of the cells) were tested for their ability to form plaques in BS-C-1 cells. A significant reduction (greater than 100-fold for IFN-β) in the release of infectious virus from the IFN-treated cells was observed at 18, 48, and even 72 h postinfection (Table 1). These results demonstrate that in IFN-treated cells, at times when infectious virus release from cells is reduced more than 100-fold, the major capsid polypeptides of HSV are being synthesized normally, which is consistent with the electron microscopic observation of assembled virus cores in such treated cells.

Synthesis of virus-specific glycoproteins in IFN-treated human cells. Two possibilities could be envisioned to explain the lack of core envelopment and transport in IFN-treated cells. First, IFN treatment could cause a reduction in the fluidity of the nuclear membrane and thus prevent normal HSV morphogenesis. We have shown previously that IFN-α and IFN-β reduce the fluidity of cellular membranes, and while this has been most definitively demonstrated at the plasma membrane (4, 19, 24), similar changes may also occur at other membranes. Second, viral glycoprotein insertion into the inner nuclear membrane may be essential for core recognition and envelopment in a similar manner to that postulated for the vesicular stomatitis virus G protein. A block in the synthesis or migration or both of such proteins would thus interfere with virus assembly. Since the immunoblotting experiments with rabbit anti-HSV-1 antiserum specifically provided no information on virus glycoprotein expression, we next analyzed the same cell lysates for the status of gB and gD by using monospecific antiserum against these proteins. The IFN-β-treated cell lysates (18 and 48 h postinfection) prepared earlier were subjected to polyacrylamide gel electrophoresis, and the fractionated proteins were transferred to nitrocellulose paper. Two sets of blots were prepared; one set was reacted with rabbit anti-gB antiserum, whereas the other was reacted with rabbit anti-gD antiserum. The results of this experiment show that unlike the levels of the capsid proteins, the levels of gD in the lysates at both periods were significantly reduced by IFN-β (Fig. 5a). The amount of gB was also significantly inhibited at 18 h postinfecion, but after further incubation (to 48 h) the levels of this protein rose to those of untreated cells (Fig. 5b). More importantly, both the pre-gB and the mature gB could be seen in the lysates, indicating that intracellular transport of gB (at least to the Golgi complex) was occurring normally. Similar experiments with IFN-α also demonstrated a reduction in the amount of gB and gD in the lysates, although not as significant as that observed after IFN-β treatment.

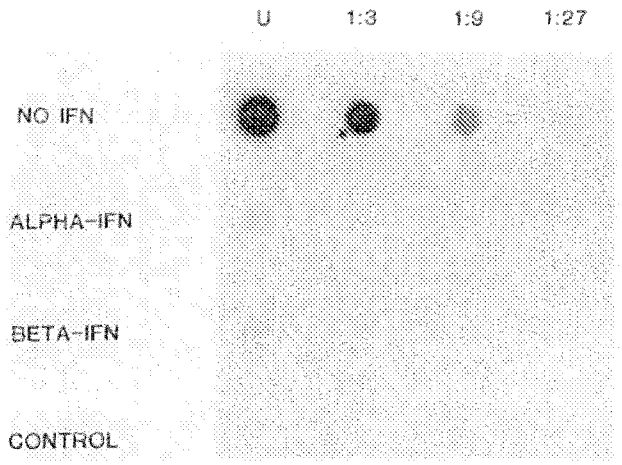
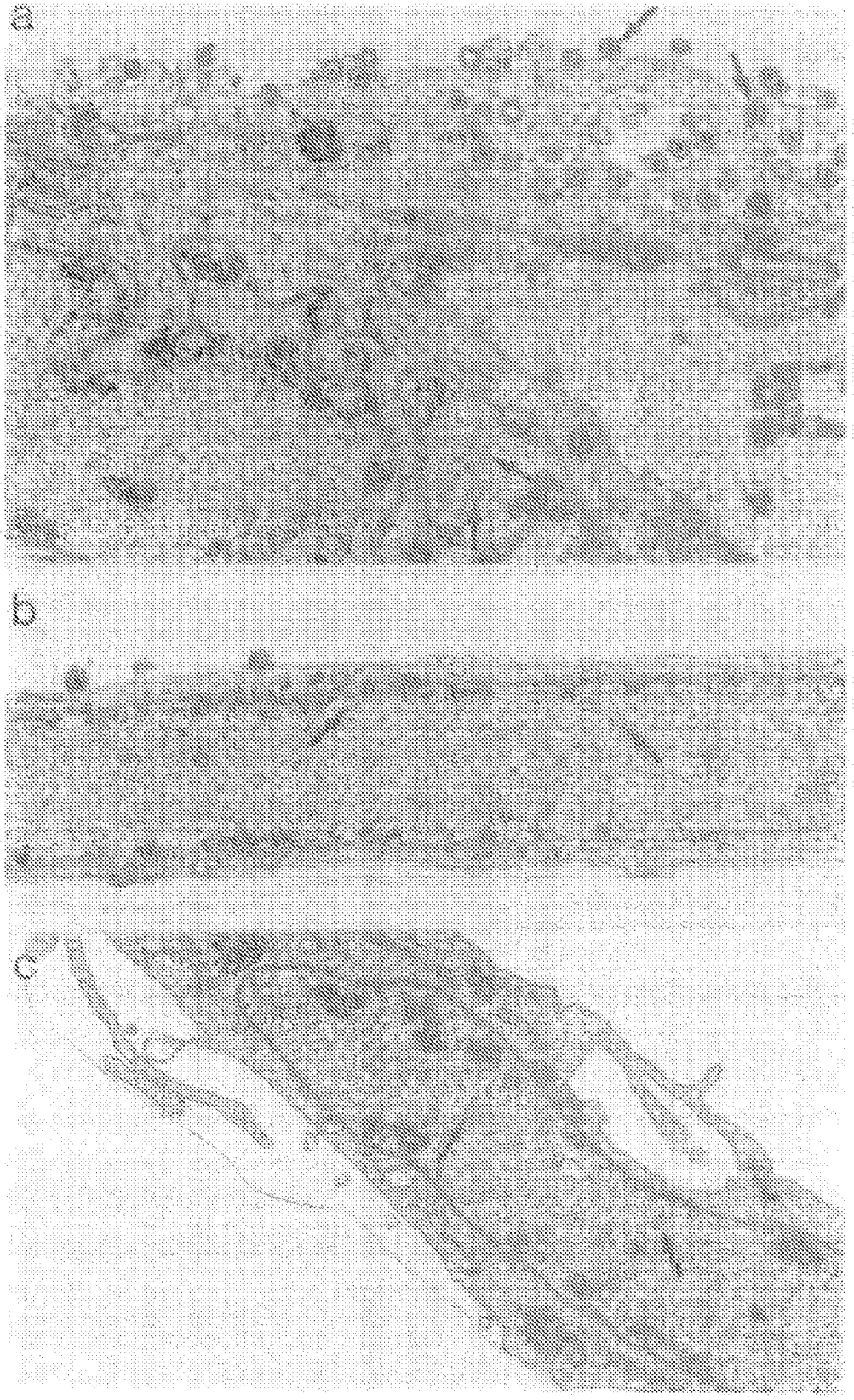


FIG. 2. Effect of cloned human IFNs on the release of extracellular virus particles. Human fibroblast cells pretreated with IFN-α_2 or IFN-β were infected with the MP strain of HSV-1 as described in the text. One set of cells served as an uninfected control. Supernatant fluids from each set were collected and clarified, and any virus present was pelleted by centrifugation. Viral DNA was extracted, serially diluted, and then processed for DNA dot blot hybridization as described in the text.

DISCUSSION

Little is known about the exact molecular mechanisms by which IFN prevents DNA virus replication. The results described in this communication, however, clearly demonstrate the stage(s) at which HSV replication is blocked by IFN. We have shown that both cloned IFN-α_2 and IFN-β significantly inhibit the replication of HSV-1 in human cells. Similar results (with IFN-α) have also been reported by Fish et al. (7), but the specific stage at which replication was affected by IFN in those experiments was not described. We show here that the inhibition of virus replication involves a block at a late stage in HSV morphogenesis, specifically at the level of virus core exit from the nucleus of IFN-treated cells. This conclusion is based on the fact that no extracellular particles were released from the IFN-treated cells, and electron microscopy demonstrated the assembly of nucleocapsids inside the nucleus of the IFN-treated cells. Consistent with these observations, IFN-treated cells showed no major differences in the nucleocapsid protein profile compared with that of untreated cells at times when the replica-



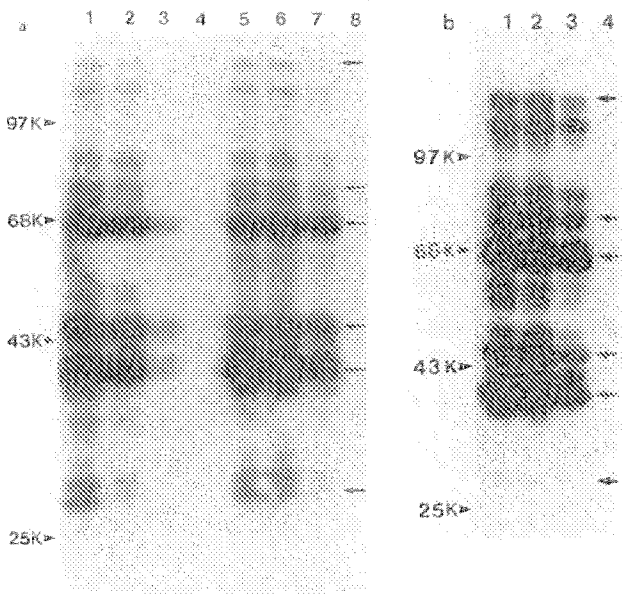


FIG. 4. Effect of cloned human IFNs on the synthesis of HSV-1 specific proteins in IFN-treated and -untreated human cells. (a) Human fibroblast cells were pretreated with 100 U of either IFN-α_2 or IFN-β per ml and then infected with the MP strain as described in the text. One set of cells served as an uninfected control. Cell lysates collected 18 and 48 h postinfection were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently processed for immunoblotting. The blot was reacted with rabbit anti-HSV-1 antiserum. Lanes: 1 through 4, 18 h postinfection; 5 through 8, 48 h postinfection; 1 and 5, no IFN; 2 and 6, INF-α; 3 and 7, IFN-β; 4 and 8, uninfected control. The major capsid proteins are indicated by arrows. (b) The experimental procedure was essentially same as described above, except that strain F of HSV-1 was used instead of strain MP and the cell lysates were collected at 24 h postinfection. Lanes: 1, no IFN; 2, IFN-α; 3, IFN-β; 4, control. The major capsid proteins are indicated by arrows.

tion of infectious virus was still inhibited more than 100-fold. The presence of virus-specific proteins in the IFN-treated cells also ruled out the possibility that the block was at the stage of virus penetration or at an early stage before viral DNA synthesis. This is in agreement with our previous finding, when we reported that similar levels of viral DNA were synthesized in IFN-treated and untreated cells. Since it has been postulated that viral glycoproteins might act as nucleation points for virus budding and envelopment in several enveloped virus systems, we investigated, through the use of monospecific antisera to two HSV glycoproteins, whether any specific effects on glycoprotein synthesis could be observed. Surprisingly, both gB and gD are present in significantly reduced amounts at 18 h postinfection in IFN-β-treated cells infected with HSV-1, and while the levels of gB appear to recover in the next 30 h, those of gD still remain significantly reduced. This effect on viral glycoprotein biosynthesis could also explain the drastic reduction in cell-to-

TABLE 1. Effect of cloned human IFN-α_2 and IFN-β on replication of HSV-1 as a function of time[a]

Incubation time (hr)	Addition	% Inhibition
18	None	0
	IFN-β (100 U/ml)	>99
	IFN-α_2 (100 U/ml)	>99
48	None	0
	IFN-β (100 U/ml)	>99
	IFN-α_2 (100 U/ml)	>94
72	None	0
	IFN-β (100 U/ml)	>99
	IFN-α_2 (100 U/ml)	>87

[a] Human cells were pretreated with different IFNs and infected with the MP strain of HSV-1 as described in the text. Supernatant fluids were collected 18, 48, and 72 h postinfection and tested for their ability to form plaques in BS-C-1 cells.

cell fusion observed in IFN-treated cells (5), since Noble et al. (17) have recently shown, through the use of monoclonal antibodies to gB and gD, that gD is the HSV-1 protein responsible for cell fusion activity. An effect of IFN on the viral glycoproteins thus could have a pleotropic action on both viral assembly-morphogenesis and cell fusion. At present, it is not possible to distinguish between a reduction in the rate of synthesis of gD or an increased rate of turnover of gD, since immunoblotting provides only a measure of steady-state levels of the protein. Pulse chase immunoprecipitation studies and an analysis of gD mRNA levels should provide information on the molecular mechanisms involved in the cause of its reduced levels.

The mechanism of IFN action on HSV replication is clearly quite different from that in other viral systems. The well-documented stimulation of a 2,5 oligoadenylate-activated endonuclease by IFNs and a protein kinase that appears to be effective in blocking translation of certain RNA viral proteins (1, 12) does not appear to be important in this case, since high levels of viral protein synthesis are observed in IFN-treated cells. Moreover, the block appears to be different from that observed in certain retroviruses, in which virus particles are assembled and enveloped in the presence of IFN but are inhibited at the stage of virus release from the cell (20; Chatterjee and Hunter, Meet, RNA Tumor Viruses 1983). We have not observed HSV cores partially enveloped by the nuclear membrane in IFN-treated cells; they appear instead to be scattered randomly through the nucleus. An effect of IFN on viral glycoprotein biosynthesis has been reported by Maheshwari and Friedman (13), who demonstrated that in mouse L cells treated with low levels of IFN, the vesicular stomatitis virus G protein was not glycosylated, but was synthesized and incorporated into virus particles, an event not observed in our studies with HSV-1. It is possible that IFN affects a unique step in the complicated transcription-translation cascade that regulates HSV-1 gene expression and in this way prevents normal expression of gB and gD. Two papers reporting conflicting

FIG. 3. Electron microscopic observations of virus particles present in thin sections of IFN-treated and -untreated human cells infected with HSV-1. (a) Thin section of HFS cells infected with the MP strain without IFN treatment, showing mature HSV particles (arrows). (b) and (c) Thin sections of HFS cells pretreated with either cloned IFN-α_2 or IFN-β, respectively, and then infected with the same strain. Note the distinct HSV cores assembled in nucleus (arrows). Magnification, ×28,000.

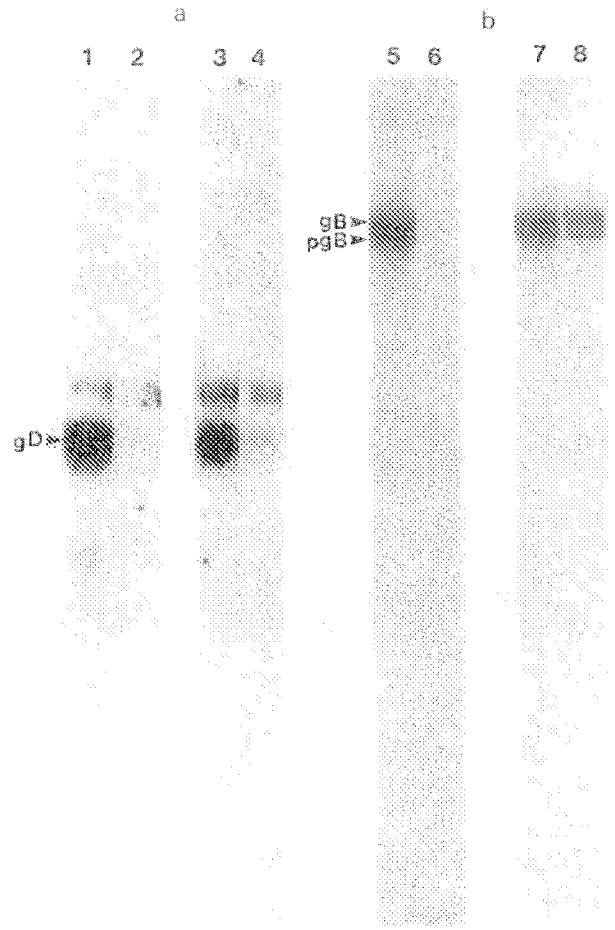


FIG. 5. Effect of cloned human IFN-β on the synthesis of gB and gD in IFN-treated and -untreated human cells. The IFN-β-treated cell lysates used in the experiment described in Fig. 4a were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in duplicate and subsequently processed for immunoblotting. The blots were reacted with either rabbit anti-gD (a) or rabbit anti-gB antiserum (b). Lanes: 1, 2, 5, and 6, 18 h postinfection; 3, 4, 7, and 8, 48 h postinfection; 1, 3, 5, and 7, no IFN; 2, 4, 6, and 8, IFN-β.

results have recently been published on the effects of IFN on HSV-1 replication. Gloger and Panet (8) observed an inhibition of immediate-early (α) polypeptide biosynthesis and a subsequent reduction of early (β) and late (γ) gene products. While those authors used significantly higher levels of IFN (2,000 U/ml), they analyzed polypeptide biosynthesis at 5 h postinfection and may have observed the delay in polypeptide biosynthesis we observed with IFN-β. However, the concentration of IFN used in our studies had no significant effect on cellular viability. Munoz and Carrasco (15), on the other hand, report no effect of natural human IFN-α on HSV-1 polypeptide synthesis or virus assembly, but rather the release of normal levels of noninfectious virus particles. We estimate from DNA dot blot experiment that less than 5% of the normal levels of virus particles were released in our experiments. While it is not possible to reconcile the results of the two studies at present, it should be noted that we have consistently observed a 10-fold-greater inhibition of infectious virus when using 4-fold less IFN than reported by Munoz and Carrasco (15). The differences in our results may thus reflect the differences in the IFN preparations. While the experiments described here clearly show that the level of expression of HSV-1 glycoproteins is significantly reduced in IFN-treated cells, we cannot at present conclude that this represents the block in viral assembly. The level of other HSV-1 gene products not quantifiable in the current experiments may also be affected in IFN-treated cells, and it could be these gene products that are required for normal nucleocapsid assembly and transport. Alternatively, membrane fluidity changes, which clearly occur in IFN-treated cells (4, 19, 24), could play a role in the disruption of HSV morphogenesis. Experiments aimed at addressing these possibilities are at present under way.

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